AD_____

Immunochemical Investigations of Cell Surface Antigens of Anaerobic Bacteria

FINAL REPORT

(for the period of 2/1/74-12/31/77)

Dennis L. Kasper, M.D.

October 15, 1984

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-74-C-4056

Harvard Medical School
Boston, MA 02118

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A161 878
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Immunochemical Investigations of Cell Surface Antigens of Anaerobic Bacteria	5. TYPE OF REPORT & PERIOD COVERED Final Report 1 Feb. 74 - 31 Dec. 77)
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Dennis L. Kasper, M.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-74-C-4056
9. PERFORMING ORGANIZATION NAME AND ADDRESS Harvard Medical School Boston, MA 02118	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102B.3M161102BS01.00.001
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701-5012	12. REPORT DATE October 15, 1984
	13. NUMBER OF PAGES 17
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Bacteroides, Bacteroides fragilis, Bacteroides fragilis ss. fragilis, anaerobic bacteria, capsular polysaccharide, lipopolysaccharides, vaccines, intra-abdominal sepsis, abscesses.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Among the anaerobic bacteria responsible for human infection, Bacteroides fragilis is the most important. This organism is particularly important in intra-abdominal sepsis or bacteremia. Several studies were conducted to determine whether any unique bacterial components of Bacteroides fragilis could account for its enhanced virulence. (cont'd)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. (cont'd)

B. fragilis has chemically incomplete lipopolysaccharides as compared with the lipopolysaccharides (endotoxins) of aerobic bacteria, and the lipopolysaccharides of Bacteroides lack the biologic potency characteristic of endotoxin. This inactivity may account for the very infrequent occurrence of disseminated intravascular coagulation or purpura that can accompany sepsis due to these organisms. Furthermore, strains of B. fragilis have capsular polysaccharides. In an animal model of intra-abdominal sepsis, the encapsulated strains caused abscesses when given without other organisms, but abscess formation from unencapsulated strains of Bacteroides generally required the administration of a synergistic aerobe. The abscesses caused by encapsulated strains were shown to be directly attributable to the capsular polysaccharide, which is an important virulence factor of this organism. Experimental animals infected with B. fragilis develop antibodies to the capsular polysaccharide, and these antibodies can be detected in a radioactive antigen-binding assay.

SUMMARY

Anaerobic bacteria are a major cause of wound related infection in military personnel. Of the various anaerobic bacteria, Bacteroides fragilis is most frequently associated with serious infections. The studies done under the support of this contract were to develop an understanding of the antigens of this organism, in an initial attempt to determine the feasibility for the development of a vaccine.

Studies were done isolating the outer membrane of this microbe and defining its major antigens. The outer membrane contained a lipopolysaccharide, proteins and a capsulr polysaccharide. Studies of the lipopolysaccharide (LPS) showed it to be biologically and chemically distinct from the LPS of aerobic gram negative bacteria. It lacked certain critical sugars and biologically it was not endotoxic.

Studies were directed to the study of the capsular polysaccharide. This complex carbohydrate was shown to be a true virulence factor and was responsible for the induction of abscesses in an animal model of intra-abdominal sepsis. Experimental animals infected with B. fragilis developed antibodies which were detectable in a radioactive antigen binding assay. It is our belief that this antigen would potentially serve as an excellent vaccine candidate. We suggest further studies to look at the protective efficacy of this antigen on the animal model of intra-abdominal sepsis. This would be the necessary prerequisite to vaccine studies in human volunteers.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals,", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

The virulence properties of bacteria have been attributed to a number of substances that appear to help the organism invade tissues. Although many bacteria elaborate virulence-enhancing substances, such as enzymes and toxins, in others the pathogenic properties are structurally integrated into the cell wall. For example, the virulence of bacteria that behave as extracellular parasites is often caused, at least in part, by antiphagocytic polysaccharide capsules that form a gel-like matrix around the cell. When the Streptococcus pneumoniae was studied in mice, it was found that unencapsulated strains were virtually avirulent because they are readily phagocytosed, but encapsulated strains were highly virulent because of their resistance to phagocytosis. Similarly, the virulence of gram-negative bacteria is thought to be caused, in part, by the presence of endotoxins in the outer membrane. Endotoxins are complex lipopolysaccharides (LPS), whose toxicity resides in the lipid A portion of the molecule, and these lipids have numerous direct biological effects. In animals LPS cause fever, transient leukopenia followed by leukocytosis, hyperglycemia, purpura, and the Shwartzman phenomenon. In addition, large i.v. doses result in irreversible shock. Our studies were concerned with the capsular polysaccharide and LPS components of anaerobic bacteria and the role of these substances in the development of infection.

Clinical and Microbiologic Aspects of Bacteroides

Among the diseases commonly caused by anaerobes are brain abscess, chronic sinusitis, dental infections, aspiration pneumonitis, lung abscess, empyema, liver abscess, intra-abdominal sepsis, and infections of the female genital tract. When optimal bacteriologic techniques are used, anaerobic bacteria can be isolated from 70-95% of patients with these diseases. A common denominator in this seemingly diverse array of septic processes is that the source of the bacteria responsible is the patient's own microflora. Thus, the anaerobic bacteria that are isolated from infected sites are normal inhabitants of the oral cavity, gastrointestinal tract, lower female genital tract, or skin. The pathogenic mechanism is usually a disruption of anatomic barriers and of other host-defense mechanisms that allows the bacteria that colonize mucocutaneous surfaces to gain access to normally sterile sites.

The bacteriology of these infections might be expected to reflect the flora of the source of the inoculum. Yet, although >400 bacterial species reside in the colon and >200 are thought to colonize healthy oral cavities, the average number of bacterial species in infections associated with colonic perforation is five, the average in dental infections is six, and for aspiration pneumonia the average is three. The anaerobic bacteria that dominate in these types of diseases include Bacteroides fragilis, Bacteroides melaninogenicus, Fusobacterium nucleatum, Clostridium perfringens, Peptostreptococcus anaerobius, and Peptococcus asaccharolyticus - six species that probably account for the great majority of anaerobic isolates in clinical laboratories. Thus, from a seemingly endless array of anaerobic bacteria in the normal flora, only a few are common in septic processes; it is likely that virulence is an important factor in their selection.

Of all the anaerobes, B. fragilis is the most frequently encountered in intra-abdominal sepsis or bacteremia. Members of the genus Bacteroides were second only to Escherichia coli as a cause of gram-negative septicemia in patients at the Mayo Clinic (Rochester, MN), and 78% of these Bacteroides are

B. fragilis. Studies of intra-abdominal sepsis and infection of the female genital tract indicated that B. fragilis is the most common cause of bacteremia in these clinical settings. Much of our work is focused on this common anaerobe.

Organisms classified as B. fragilis were formerly subdivided into six subspecies: fragilis, distasonis, vulgatus, thetaiotaomicron, ovatus, and an unspecified group, subspecies "other." These subspecies share many phenotypic characteristics, including resistance to penicillins, and their separation was based on minor variations in biochemical reactions. Although they have been reclassified into distinct species on the basis of studies of DNA homology, the older, more familiar classification is used in this presentation.

The distribution of the B. fragilis subspecies is markedly different in normal flora and infected sites. In the colon, the usual source of B. fragilis in septic processes, the numerically dominant subspecies are distasonis, vulgatus, and thetaiotaomicron; subspecies fragilis accounts for only about 0.5% of the colonic microflora. In clinical specimens, however, subspecies fragilis is most often encountered. During a two-year period we observed 338 strains of B. fragilis in blood cultures or exudates from clinical infections. Of these organisms, 260 (78%) were subspecies fragilis. Thus, when compared to the numerical concentrations in normal flora, B. fragilis subspecies fragilis is present in a disproportionately large number of clinical isolates; its predominance in exudate and blood strongly suggests that this subspecies has unique virulence properties.

The Outer Membrane of B. fragilis

We conducted several studies to determine whether any unique bacterial components of subspecies fragilis could account for its enhanced virulence. We began with immunochemical analysis of the outer membrane structure, and this investigation provided some insight into the virulence of the components of the membrane.

In B. fragilis and other gram-negative bacteria, the cell wall consists of an outer membrane, an inner, or cytoplasmic membrane, and a rigid peptidoglycan layer, which separates the two trilaminar membranes (figure 1). The cytoplasmic membrane is generally the site of membrane synthesis and thus contains most of the biosynthetic enzymes. The outer membrane contains the antigens that are encountered by the host, in other words, the surface components of the bacteria.

The outer membranes were loosened from intact B. fragilis by heating of organisms that were suspended in a buffer that contained 10 mM EDTA. This procedure was followed by gentle shearing through a 25-gauge needle and purification by differential ultracentrifugation. The purity of the membrane fraction was assessed by isopycnic ultracentrifugation on a 30-65% sucrose density gradient. A single peak was identified (figure 2); its density was 1.23 g/ml, a value similar to that reported for other bacterial outer membranes.

The outer membrane in gram-negative bacteria contains protein, LPS, and loosely bound lipids. Capsular polysaccharide is also present in some bacteria, including B. fragilis. When we studied the protein component of the outer membranes of strains of B. fragilis by means of electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, the representative strains from each

subspecies had distinct peptide-band patterns (figure 3, left). This finding was not surprising since the outer membrane proteins in other bacteria are biochemically and genetically heterogeneous. Of greater interest was the observation that strains of subspecies fragilis had nearly identical peptide-band patterns (figure 3, right), a similarity that is unusual within a biochemical group.

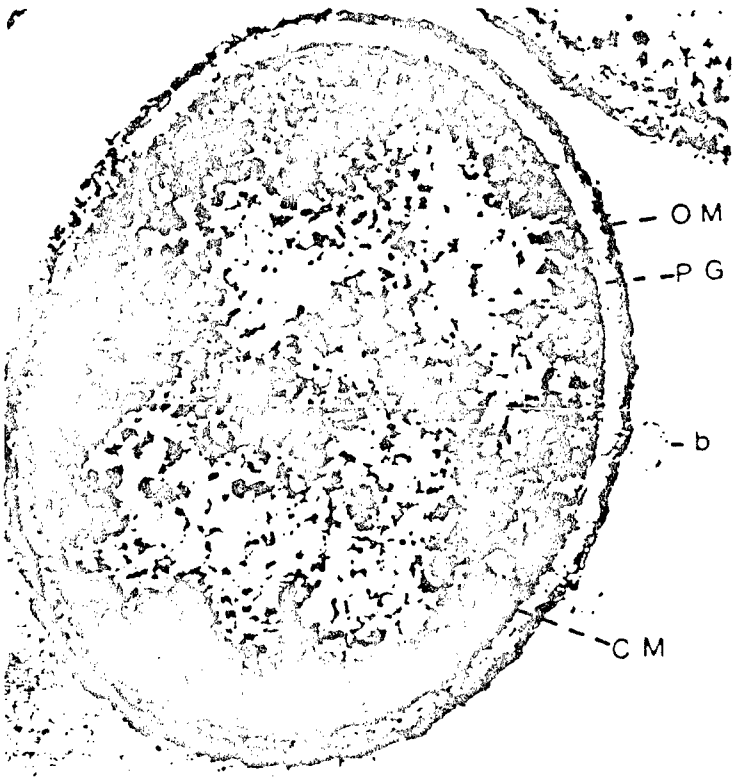

Figure 1. Electron micrograph of *Bacteroides fragilis* subspecies *fragilis* stained by standard techniques (×120,000). OM = outer membrane; PG = peptidoglycan; b = bleb of outer membrane that is extruded into the growth medium; CM = cytoplasmic membrane. The scale marker denotes 1 μm.

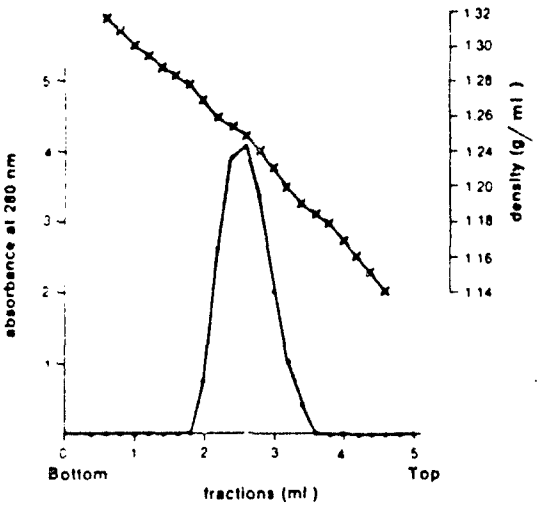

Figure 2. Absorption at 280 nm of fractions collected from a 30% 65% discontinuous sucrose density gradient centrifugation of the outer membrane fraction of *Bacteroides fragilis* subspecies *fragilis* (strain ATCC 25745, American Type Culture Collection, Rockville, Md.). Density (× ———— ×) was determined with a refractometer.

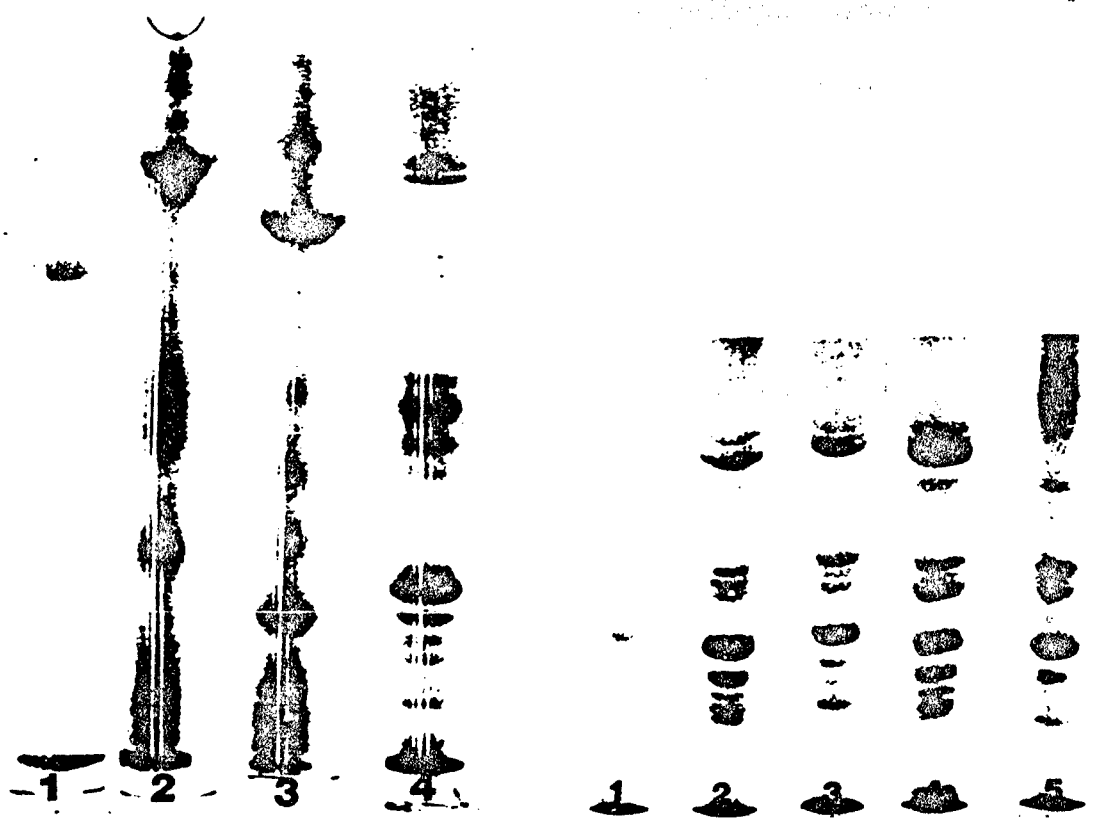

Figure 3. Electrophoretic studies of structural proteins in outer membranes of cells that represent four subspecies of *Bacteroides fragilis* (*left*) and of cells that represent five strains of *B. fragilis* subspecies *fragilis* (*right*). Electrophoresis was performed in sodium dodecyl sulfate-polyacrylamide gels. The subspecies shown in the figure on the left are *distasonis* (strain 8503), *vulgatus* (strain 8482), *thetaiotaomicron* (strain 12290), and *fragilis* (strain 23745). The strains of subspecies *fragilis* shown in the figure on the right are 2429 (Los Angeles, Calif.), ATCC 23745 (American Type Culture Collection, Rockville, Md.), 1262 (Philadelphia, Pa.), 2244 (Los Angeles, Calif.), and 26747 (Boston, Mass.). These strains have nearly identical peptide-band patterns.

As stated above, the LPS component is regarded as a major virulence factor of gram-negative bacteria. However, the LPS of B. fragilis is biologically distinct from that of aerobic gram-negative bacteria. Although, chemically, it is a lipopolysaccharide, it does not function as an endotoxin. The LPS of aerobic gram-negative bacteria contains a lipid (lipid A) portion (figure 4), which consists primarily of a disaccharide backbone highly substituted with long-chain fatty acids. This portion is linked to a carbohydrate core, which contains two unusual sugars (2-keto-3-deoxyoctonate and a heptose), as well as glucose, galactose, and glucosamine. The core is linked to the repeating carbohydrates of the O side chain. Most aerobic gram-negative bacteria have biologically similar lipopolysaccharide cores, and antibodies to these cores are cross-reactive between bacterial species. However, the O side chain differs markedly between strains even of the same species and thus is the basis of serologic typing schemes for the Enterobacteriaceae. The core of the

major pathogenic Bacteroides species (fragilis and melaninogenicus) is quite distinct from the core of other facultative and of at least some anaerobic bacteria.

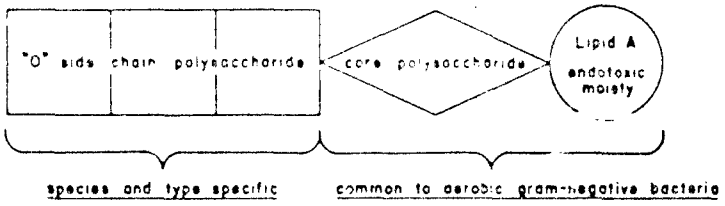

Figure 4. Schematic diagram of the typical lipopolysaccharide or endotoxin of gram-negative bacteria.

Studies on an Animal Model of Intra-abdominal Sepsis

Animal models have proved to be valuable research tools for the study of infectious diseases. Many of these models have become an integral part of studies dealing with bacterial virulence and therapeutic efficacy. In recent years, animal models have been used to document the role of various microbial species in mixed infections, including infections with combinations of facultative and anaerobic organisms. In many instances, knowledge gained from studies employing animal models for infection has led to a better understanding of the human disease process.

The rat model of intra-abdominal sepsis, was originally devised to determine whether the obligate anaerobes that are part of the intestinal microflora played any role in the infectious process associated with intra-abdominal sepsis. These early studies resulted in the finding that certain obligate anaerobes, such as Bacteroides fragilis, were an essential part of the experimental infection. Subsequent studies were first directed toward antimicrobial efficacy and then focused on the virulence factors of B. fragilis. This model, along with several modifications, is currently being used to determine which factors are important to host immune response. A summary of these previous findings illustrates how this animal model was employed to provide data regarding a specific infectious process. During these studies considerable information has been derived addressing the more basic issues of bacterial virulence and host immune response.

Rat Model for Intra-abdominal Sepsis

The initial studies with Wistar rats were designed to develop an animal model simulating intra-abdominal sepsis as described for human patients. The goal of these early experiments was to evaluate the role of the various microbial species in infections resulting from contamination of the peritoneum with the microbial milieu present in the large intestine. Preliminary studies indicated that cecal contents of grain-fed rats did not harbor the same microbial species

present in human intestinal contents. However, placing rats on a diet of lean ground beef for a two-week period resulted in alteration of the cecal microflora to one more compatible with that noted in human intestinal contents. Once an appropriate inoculum had been derived, subsequent experiments employed an inoculum of pooled cecal contents from meat-fed rats and $BaSO_4$ contained within gelatin capsules, which were surgically implanted into the peritoneal cavity of 180 g Wistar rats.

The results of these early experiments indicated that a biphasic disease process occurred. Rats initially developed an acute peritonitis associated with a death rate of 40%. Although surviving recipients appeared healthy within seven days of implantation, these animals were shown to have uniformly developed intra-abdominal abscesses when necropsied at 14 days. Bacteriologic evaluation of peritoneal exudates and abscess contents of implanted animals also revealed two distinct phases of the experimental infection. During the acute-peritonitis phase of disease, Escherichia coli and enterococci were the numerically dominant species. In contrast, abscess contents yielded a Bacteroides species (not B. fragilis) and Fusobacterium varium as the most numerous species. The data indicated that facultative species such as E. coli were important to the initial phase of the experimental infection and that obligate anaerobes were more important to the abscess phase of the infection.

Subsequent experimentation confirmed these initial observations by two different methods. First it was shown that suppression of the coliform population with gentamicin resulted in a decrease in the number of deaths during the peritonitis phase, and suppression of the anaerobic microbial population with clindamycin decreased the occurrence of abscesses. Second, it was shown that a simplified inoculum containing only two species required the presence of E. coli for death to occur and either the Bacteroides species or F. varium, isolated during previous experiments, for abscess to occur. Elimination of either E. coli or the two principal anaerobes from the implanted inoculum prevented the development of the biphasic infection. It was also shown that no single species implanted alone resulted in abscess formation; this suggested that a synergistic relationship between anaerobes and facultative species was necessary for abscess formation.

The conclusions made from these studies were that both facultative and obligately anaerobic microorganisms were involved in intra-abdominal sepsis. Facultative species, such as E. coli, were responsible for the mortality associated with the peritonitis phase of disease. However, intra-abdominal abscess formation - the later, more indolent part of the experimental infection - required the presence of an obligate anaerobe, such as F. varium or Bacteroides species, in combination with a facultative species.

Application of the Rat Model to Studies of Virulence of B. fragilis

Among the many species of obligate anaerobes associated with clinical infection, B. fragilis has received considerable attention because of its dominance in human intra-abdominal sepsis and its resistance to certain commonly used antimicrobial agents. On the basis of the findings of Kasper and Seiler and Kasper et al. that B. fragilis contains a polysaccharide capsule not present on phenotypically similar organisms - the rat model was modified to

determine whether this microbial species was more virulent than other phenotypic similar, but unencapsulated, strains. From our previous studies we knew that abscess formation with a Bacteroides species (formerly B. fragilis subspecies "other") from rat cecal contents required the presence of a facultative species. A comparison of the encapsulated species, B. fragilis, to other unencapsulated Bacteroides species was made utilizing the various strains alone and in combinat with enterococci (known to potentiate abscesses in conjunction with an unencapsulated Bacteroides species). It was shown that both encapsulated B. fragilis and unencapsulated members of the B. fragilis group (Bacteroides distasonis, Bacteroides vulgatus, Bacteroides thetaiotaomicron and Bacteroides ovatus) were capable of provoking abscesses when implanted into rats in combination with sterile cecal content $BaSO_4$, and enterococci. None of the unencapsulated strains, by themselves, were capable of causing abscesses. However, B. fragilis implanted alone was capable of causing abscesses in 95% of implanted animals in the absence of other viable bacteria. More important, heat-killed B. fragilis alone was capable of provoking abscesses in 65% of recipients in the absence of any viable bacteria. These findings suggested that B. fragilis produced a heat-stable substance that could produce abscesses and that this substance was not present on unencapsulated strains. Studies were therefore conducted using the various outer membrane components of the B. fragil cell, including the capsular polysaccharide, outer membrane, lipopolysaccharide, and protein polysaccharide fractions. The results of these experiments showed that the fraction responsible for abscess formation when implanted into Wistar rats was the capsular polysaccharide.

Antibody response to the B. fragilis capsule in the rat peritonitis model

The humoral antibody response to the capsular polysaccahiride of B. fragilis was quantitated in animals with intra-abdominal abscesses by means of a sensitive, radioactive antigen-binding assay. The antibody detected by this technique correlated highly with that measured by quantitative precipitin analysis ($r = 0.943$). Animals infected with encapsulated B. fragilis had high levels of circulating serum antibody to the capsular polysaccharide (figure 5). This antibody could be induced by implantation of live organisms, heat-killed organisms, heterologous strains of B. fragilis, or various outer-membrane components that contained the capsular antigen, and the immunogenicity of the capsular polysaccharide could be enhanced by complexing to the outer membrane or to outer-membrane proteins.

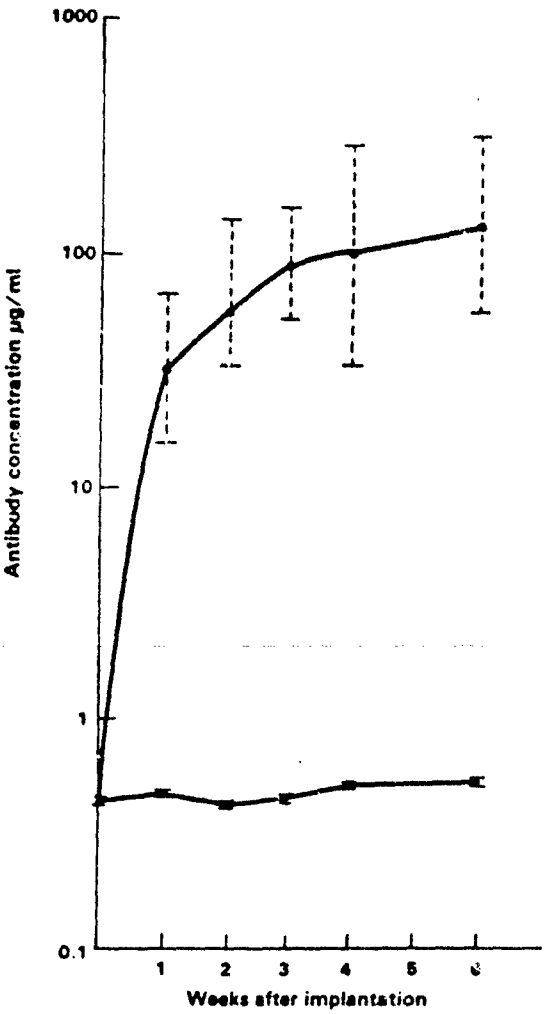

Figure 5 . Quantitative antibody response to the capsular polysaccharide of *Bacteroides fragilis* for groups of 10 rats implanted with encapsulated *B. fragilis* (line with dashed brackets) and unencapsulated *B. fragilis* (line with solid brackets). Means ± SD are plotted for each weekly sampling of blood after implantation (week zero). Implants for both groups included 5×10^7 viable enterococci, sterile cecal contents, $BaSO_4$, and either the encapsulated *B. fragilis* or the unencapsulated strain of *Bacteroides*.

REFERENCES CITED

1. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 193:265, 1951.

2. Kasper DL, Hayes ME, Reinap BG, Craft FO, Onderdonk AB, Polk BF. Isolation and identification of encapsulated strains of Bacteroides fragilis. J Infect Dis. 136:75-81, 1977.

Publications DAMD17-74-C-4056
2/1/74-12/31/77

1. Kasper DL, Seiler MW. Immunochemical characterization of the outer membrane complex of Bacteroides fragilis subspecies fragilis. J Infect Dis. 132:440-450 1975.

2. Kasper DL, The polysaccharide capsule of Bacteroides fragilis subspecies fragilis: Immunochemical and morphologic definition. J Infect Dis. 133:79-87, 1976.

3. Kasper DL. Chemical and bilogical characterization of the lipopolysaccharide of Bacteroides fragilis subspecies fragilis. J Infect Dis. 134:59-66, 1976.

4. Polk, BF, Kasper DL. Bacteroides fragilis subspecies fragilis in clinical isolates. Ann Intern Med. 86:569-571, 1977.

5. Mansheim BJ, Kasper DL. Purification and immunochemical characterization of the outer membrane complex of Bacteroides melaninogenicus subspecies asaccharolyticus. J Infect Dis. 135:787-799, 1977.

6. Onderdonk AB, Kasper DL, Cisneros RL, Bartlett JG. The capsular polysaccharide of Bacteroides fragilis as a virulence factor: Comparison of the pathogenic potential of encapsulated and unencapsulated strains.

7. Kasper DL, Hayes ME, Reinap BG, Craft FO, Onderdonk AB, Polk BF. Isolation and identification of encapsulated strains of Bacteroides fragilis. J Infect Dis. 136:75-81, 1977.

8. Kasper DL, Onderdonk AB, Bartlett JG. Quantitative determination of the antibody response to the capsular polysaccharide of Bacteroides fragilis in an animal model of intra-abdominal abscess formation. J Infect Dis. 136:789-79 1977.

9. Mansheim BJ, Onderdonk AB, Kasper DL. Immunochemical and biologic studies of the lipopolysaccharide of Bacteroides melaninogenicus subspecies asaccharolyticus. J Immunol. 120:72-78, 1978.

DISTRIBUTION LIST

12 copies	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, DC 20307-5100
4 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, Maryland 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100